Towards an efficient and safe fertility preservation strategy in boys facing chemo-and radiotherapy

Ellen Goossens and Herman Tournaye

Abstract

Thanks to chemo- and radiotherapy the life expectancy for children with cancer has increased considerably. However, these cancer treatments may have serious side effects, sterility being an important one. Adult men have the possibility to circumvent this side effect by banking their semen. Prepubertal boys, however, can not benefit from this option, because spermatogenesis has not been initiated yet. A possible solution to preserve their fertility is banking and transplanting the precursor cells of spermatogenesis, i.e. the spermatogonial stem cells or SSCs. Since the first report of this model in 1994, a lot of progress has been made. However, before SSC transplantation (SSCT) can be offered in a clinical setting, both the efficiency and safety of the technique has to be guaranteed. This review overviews the studies that have been carried out in this regard.

Keywords: cancer, cell therapy, infertility, spermatogonia, transplantation.

INTRODUCTION

About one in every 600 children will develop cancer before the age of 15 years. Thanks to the remarkable progress that has been made in the treatment of cancer in infants and children more than 80% of them can now be cured. However, not only the malignant cells are destroyed by the cancer treatment, also the rapidly dividing testicular spermatogonial stem cells (SSCs) can be damaged or lost. As a result, spermatogenic failure and infertility may occur. Especially boys who need aggressive treatments in
combination with bone marrow transplantation are at risk for lifelong sterility. Besides cancer, other diseases requiring gonadotoxic treatments (e.g. sickle cell disease) or genetic diseases (e.g. Klinefelter’s syndrome, Yq-deletions) may lead to SSC loss.

It has been estimated that, by now, one in 250 adults in the age group of 20–30 years old is a childhood cancer survivor (1, 2). A case–control study indicated that 30% (10 patients out of 33) of male childhood cancer survivors were azoospermic at adolescent or adult age (3). The inability to father genetically own children might have high impact on the psychological well-being of the patient in later adulthood.

It is clear that prevention of sterility needs special attention in both oncology and reproductive medicine. For adult men facing a sterilizing cytotoxic treatment, banking of sperm before treatment has become routine practice. However, no such prevention is possible before puberty since no active spermatogenesis is present. In female patients, preventive cryopreservation of ovarian tissue has been performed and recently even led to live births after grafting (4, 5). Similar to this approach, the preservation of prepubertal testicular tissue might provide a potential solution to young cancer patients. After being treated, frozen-thawed SSCs are to be transplanted back to the patient in order to re-establish spermatogenesis (6) (Fig. 1).

**Figure 1. SSC banking and transplantation as a method for fertility preservation/restoration in prepubertal boys facing chemo- or radiotherapy.** Before the start of the gonadotoxic treatment, either a testicular biopsy or an orchidectomy is performed in the prepubertal boy. The tissue is cut into fragments of about 6 mm³ and frozen as tissue pieces. The SSCs are stored in liquid nitrogen during the time of therapy and recovery. When the boy is in full remission, the tissue fragments can be thawed, digested and transplanted back to the remaining testis. Ideally, these stem cells re-colonize the seminiferous tubules and reinitiate spermatogenesis, leading to mature spermatozoa and thereby restoring fertility in the patient.
SSCs are undifferentiated germ cells that balance self-renewal and differentiation to maintain spermatogenesis throughout adult life. This is a productive stem cell system that produces millions of spermatozoa each day. In rodents, SSCs are presumed to be the A-single (Aₕ) spermatogonia and probably some A-paired (Aₚϕ) spermatogonia. These spermatogonia are located on the basal membrane of the seminiferous tubules. More differentiated cells [A-aligned (Aₐal) and B-spermatogonia] exist as larger chains (4-32 cells) connected by intercellular bridges (7, 8). In the primate and human testis, two cell types can be distinguished on the basal membrane of the seminiferous tubules, A-dark (Aₐd) and A-pale (Aₚp) spermatogonia. It is assumed that Aₚd represents the reserve stem cells and Aₚp the active stem cells. Under normal circumstances, active Aₚp proliferation maintains spermatogenesis by balancing the production of differentiating B-spermatogonia and by renewing the Aₚp pool (9-11). Aₚd spermatogonia only divide in reaction to damage to the testis (12).

The technique of SSCT was first reported in mice by Brinster’s group in 1994. It involves the introduction of a germ cell suspension from a fertile donor testis into the seminiferous tubules of an infertile recipient mouse (13). Transplanted SSCs are able to relocate onto the basement membrane and colonize the tubules during the first month after transplantation. From that moment on, SSCs start to proliferate and initiate spermatogenesis. The first meiotic germ cells appear after one month and their number gradually increases thereafter (14). Recipient mice are able to reproduce in vivo after transplantation and produce transgenic offspring (15).

Subsequently, these experiments were performed using SSCs that had been frozen and thawed (16). Shortly after, this technology was adapted to other mammalian species, including primates (17-21). Even the transplantation between different species with close phylogeny was proven successful (22, 23). These encouraging results, especially those from primate studies, suggest a possibility of banking and subsequently transplanting human SSCs to prevent sterility caused by SSC loss. SSC banking and subsequent autologous transplantation after thawing could thus theoretically circumvent sterility induced by chemotherapy in pre-pubertal boys cured of cancer. However, before this application can be introduced in the clinic, the efficiency and the safety of the procedure has to be guaranteed.

THE EFFICIENCY OF SSCT

Although live offspring has been reported after SSCT, it remained unclear whether the fertilizing and developmental potential of spermatozoa obtained after transplantation is normally preserved. The few studies reported on this topic have been performed on mice. The functionality of spermatozoa obtained after SSCT were investigated by both in-vivo and in-vitro fertilization (IVF and ICSI) (24). After mating transplanted male mice with fertile females, 35% of the females with a copulating plug became pregnant. This rate was significantly lower compared to fertile couples, where this percentage reached 90%
A decrease in fertility was also seen, more in particular a decrease in litter size. Similar observations were made after IVF, where fertilization and blastocyst developmental rates were significantly lower in the transplanted group (p<0.0001). However, after ICSI, fertilization and blastocyst developmental rates were comparable to control spermatozoa (Table 1).

Spermatozoa obtained after SSCT are thus capable of fertilizing oocytes, but the efficiency of reproduction seemed impaired. Three possible reasons for this observation are 1) a lower sperm concentration, 2) a reduced sperm vitality, and/or 3) inferior sperm motility. To clarify this, we designed a study to analyze the motility characteristics of spermatozoa after SSCT (25). Compared to controls, the concentration of epididymal sperm was reduced, which was in agreement with the histology of the testis. On average, only 50% of the seminiferous tubules established spermatogenesis after SSCT, whereas almost all tubules showed spermatogenesis in control testes (24). The explanation may be found in the transplantation procedure. A maximum of 10µl of cell suspension could be injected in the mouse testis. If the cell suspension is not enriched for spermatogonia, which was the case in afore-mentioned experiment, no more than 2000 spermatogonia were injected. Since transplantation efficiency is only 12% (26), it is reasonable to expect that barely 240 spermatogonia had colonized the seminiferous tubules. This is extremely low, compared to the normal amount of 35000 (27). Sperm vitality was comparable to the controls, but sperm motility and hyperactivity of post-transplantation spermatozoa were significantly reduced. The movement pattern of the individual

<table>
<thead>
<tr>
<th>Spontaneous mating</th>
<th>No. of plugs</th>
<th>Pregnancy rate(^a) (%)</th>
<th>No. of fetuses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplanted mice</td>
<td>17</td>
<td>6 (35)(^b)</td>
<td>17</td>
</tr>
<tr>
<td>Control mice</td>
<td>10</td>
<td>9 (90)(^b)</td>
<td>76</td>
</tr>
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<table>
<thead>
<tr>
<th>IVF</th>
<th>No. of oocytes</th>
<th>Fertilisation rate(^c) (%)</th>
<th>Developmental rate(^d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplanted mice</td>
<td>154</td>
<td>88 (57)(^e)</td>
<td>24 (27)(^f)</td>
</tr>
<tr>
<td>Control mice</td>
<td>195</td>
<td>155 (79)(^e)</td>
<td>88 (57)(^f)</td>
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<th>ICSI</th>
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<tbody>
<tr>
<td>Transplanted mice</td>
<td>83</td>
<td>57 (69)</td>
<td>17 (30)</td>
</tr>
<tr>
<td>Control mice</td>
<td>62</td>
<td>38 (61)</td>
<td>14 (37)</td>
</tr>
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\(^a\)Pregnancy rate = (number of pregnancies / number of plugs) \times 100; \(^b\)p = 0.006; \(^c\)Fertilisation rate = (number of two-cells / number of oocytes) \times 100; \(^d\)Developmental rate = (number of blastocysts on day 5 / number of two-cells) \times 100; \(^e\)p < 0.00001.
spermatozoon was normal at the time of isolation, but decreased more rapidly in-vitro compared to controls. This difference reached a significant level already after three hours, which is the time necessary for an IVF procedure. The reduced fertilization rate may thus be explained by a lower number of motile spermatozoa and a decrease in movement parameters (Fig. 2).

IMPROVING TRANSPLANTATION EFFICIENCY

It has been estimated that, in the adult mouse, the testis contains only one SSC in 5000 testicular cells (28). Since the number of stem cells is crucial for the success of SSCT (29), enriching the proportion of stem cells may improve colonization efficiency and bring clinical applications closer. A commonly used method to select for SSCs is fluorescence-activated cell sorting (FACS). The first SSC markers were reported by Shinohara’s group who found that SSCs could be enriched 10-fold using antibodies against β1-integrin (CD29) and α6-integrin (CD49f) (30). Antibodies against c-KIT-receptor can be used for negative selection since its expression is limited to differentiating spermatogonia (31). During the last decade, more markers were identified. SSCs were found to be positive for THY1 (CD90), CD9, CD24, GFRα1, CDH1 (32-35) and negative for major histocompatibility complex class I (MHC-I), SCA1, CD34 and αV-integrin. Although it was possible to highly enrich cell populations for SSCs using combinations of positive and negative markers, a pure SSC

![Figure 2. Sperm parameters after SSCT in the mouse.](image)

Spermatozoa were maintained in-vitro for 24 hours. The vitality of the spermatozoa retrieved after SSCT (WW) was not different from controls (Ctrl). Sperm motility was significantly lower at the time of isolation and remained low during culture. The same is true for progressive motility. Spermatozoa were less hyperactivated after SSCT.
suspension could not be produced. So far, the highest level of SSC enrichment (200-fold) has been achieved based on Thy1 expression (32, 36). Very recently, a new marker for rodent SSCs was proposed by Oatley et al (37). Inhibitor of DNA binding 4 (ID4) has the most restricted expression pattern observed to date. However, not all single spermatogonia express this marker, suggesting for heterogeneity amongst the pool of single spermatogonia. Whether the SSC pool resides entirely in the population of single spermatogonia or extends to pairs and aligned spermatogonia still needs to be clarified (Table 2).

Human spermatogonia express many markers of rodent spermatogonia ($\alpha_6$-integrin, GFRα1, THY1). However, other markers are not shared. For example, testis-specific protein, Y-linked 1 (TSPY) is not expressed in mouse spermatogonia and $\beta$-integrin is not appropriate for humans (38). Human spermatogonia are also positive for CD133 and SSEA4 (39, 40).

Apart from selecting SSCs by the presence or absence of cell surface molecules, specific culture systems can also be used for enriching the stem cell population and/or expansion of the stem cell pool. Nagano et al. reported the first long-term survival of SSCs on STO-cell feeder layers for approximately four months (41). Suppressing SSC differentiation improved their in-vitro maintenance (42). SSCs could be kept in culture for more than five months, when glial cell-line derived neurotropic factor (GDNF), epithelial derived growth factor (EGF), basic fibroblast growth factor (bFGF) and leukemia inhibitory factor (LIF) were added to the culture medium (43). Even in the absence of any feeder layer or serum, stem cells could survive for more than six months (44) and expanded at least approximately $1.4 \times 10^{13}$-fold from the initiation of culture to 149 days (45). Several studies showed that SSC self-renewal is critically dependent on GDNF and that bFGF co-operates with GDNF for rodent SSC growth (46-49). As extrinsic factors for self-renewal are conserved among many mammalian species, culture systems developed in rodent models might be applicable to humans.

### Table 2. Commonly used surface markers for enriching rodent SSCs.

<table>
<thead>
<tr>
<th>Positive marker</th>
<th>Cell types expressing the marker</th>
<th>Negative marker</th>
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<tbody>
<tr>
<td>$\alpha_6$-integrin</td>
<td>A$_s$-spermatogonia $\rightarrow$ spermatocyte</td>
<td>$\alpha_6$-integrin</td>
</tr>
<tr>
<td>$\beta_1$-integrin</td>
<td>A$_s$ $\rightarrow$ B-spermatogonia</td>
<td>c-kit</td>
</tr>
<tr>
<td>CD9</td>
<td>A$_s$ $\rightarrow$ B-spermatogonia</td>
<td>CD34</td>
</tr>
<tr>
<td>Thy1</td>
<td>A$<em>s$ $\rightarrow$ A$</em>{pr}$ -spermatogonia</td>
<td>SCA1</td>
</tr>
<tr>
<td>GFR α1</td>
<td>A$<em>s$ $\rightarrow$ A$</em>{pr}$ -spermatogonia</td>
<td>MHC-I</td>
</tr>
<tr>
<td>CDH1</td>
<td>A$<em>s$ $\rightarrow$ A$</em>{pr}$ -spermatogonia</td>
<td></td>
</tr>
<tr>
<td>CD24</td>
<td>A$<em>s$ $\rightarrow$ A$</em>{pr}$ -spermatogonia</td>
<td></td>
</tr>
<tr>
<td>ID4</td>
<td>A$_s$-spermatogonia</td>
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Sadri-Ardekani et al. isolated and cultured testicular cells from both adult men (50) and prepubertal boys (51). They were able to demonstrate SSC self-renewal in-vitro. The expression of spermatogonial markers was maintained throughout the entire culture period. The ability to provide long-term culture and proliferation of human SSCs in-vitro is a huge step forward in the translation of SSCT to a clinical setting.

SAFETY OF SSCT

Next to the evaluation of the efficiency, the safety of SSCT needs to be investigated too before considering any implementation in the clinic. The length, weight and development of first and second generation offspring were studied in a mouse model (52). The offspring developed normally with their length and weight not being different from controls, provided that donor and acceptor mouse were genetically identical (Fig. 3).

To examine the incidence of genetic abnormalities after SSCT, karyotypes were determined in spermatozoa obtained after SSCT and in descendants of transplanted mice using array comparative genomic hybridization analysis (53). Numerical chromosomal aberrations could not be detected in spermatozoa from transplanted males, or in their offspring. The few genetic deviations (deletions, amplifications) observed turned out to be polymorphisms (Fig. 4).
Figure 4. Array comparative genomic hybridization on offspring after SSCT in the mouse. A detailed graph of the karyotype of 1st generation offspring showing the cleanlog2 ratios for each chromosome separately. Every genomic gain or loss found in the offspring could also be identified in the mother, implicating that these genetic abnormalities were in fact polymorphisms. The pink bars represent the 4xSD threshold level.
The next study aimed at evaluating epigenetic modifications. Epigenetic modifications are covalent modifications present on either the DNA itself or on the histones that are closely associated with the DNA. Both modifications are important in regulating gene expression without changing the genetic code itself. It is now well understood that a disrupted or altered epigenome can cause diseases. Germ cells present unique and potentially important epigenetic modifications. DNA methylation is usually associated with gene inactivation. The enzymes catalyzing methylation are divided into two categories: "maintenance" and “de novo” DNA methyltransferases (DNMTs). DNMT1 has a preference for hemi-methylated DNA and is critical for the maintenance of DNA methylation patterns in replicating cells. DNMT3A and DNMT3B are expressed during embryonic development and are essential for establishing new DNA methylation patterns. DNMT3A is located mainly in the heterochromatin, suggesting for a housekeeping role. A deficiency of this enzyme disrupts spermatogenesis and prevents the methylation of imprinted genes (54). Many of the imprinted genes are involved in developmental processes such as regulation of embryonic development, placental function, foetal growth, and maternal behaviour. Aberrant genomic imprinting can thus result in morphological, developmental or functional abnormalities in offspring.

The general methylation status and the expression of DNMT1 and DNMT3A after SSCT were studied in a stage-dependent manner by immunohistochemistry. No differences could be observed compared to control tissue (unpublished data). In addition, the DNA methylation pattern of three selected genes [two imprinted (Igf2 and Peg1) and one non-imprinted (α-Actin)] was studied using pyrosequencing, both in spermatozoa and live born offspring obtained after SSCT (52). Here again, no differences were observed among controls, spermatozoa obtained after SSCT and 1st and 2nd generation offspring (Fig. 5).
Figure 5. Methylation status of Peg1 (A, D, G, J), Igf2 (B, E, H, K) and α-Actin (C, F, I, L) in post-transplantation spermatozoa and offspring. Panel A, B and C represent the data obtained from liver of first and second generation offspring obtained after transplantation, while panel D, E and F represent the data for kidney and panel G, H and I show the data for placenta. The X-axis represents the analysed CpG sites (1 to 6); the Y-axis shows the methylation percentage. Blue boxes represent the data obtained from first generation offspring (n=24); red boxes show the data obtained from second generation offspring (n=14) and yellow boxes show the control data (n=12). The data obtained from spermatozoa of transplanted mice (n=5; light blue boxes), first generation offspring (n=7; red boxes) and control mice (n=10; yellow boxes) are shown in panel J, K and L. The methylation status of control and first or second generation offspring did not show significant differences.

Another important epigenetic mechanism is the modification of histones. Histones are rich in arginine- and lysine-residues, which are subject to post-translational modifications such as acetylation, methylation, sulphonation, ubiquitination or sumoylation. It is assumed that histone acetylation is a preliminary step to the histone-protamine exchange. Protamine-DNA interaction results in chromatin condensation and is important for a correct differentiation of round spermatids into mature spermatozoa. A too early acetylation of histone 4 will cause a premature condensation of the nuclei and will consequently lead to infertility (55). Histone methylation can lead to gene inactivation or gene expression depending on which amino acid is methylated and the number of coupled methyl groups. H3K4me3 is important for the progression of the meiotic prophase. An inefficient
H3K4me3 will result in sterility (56). In the human spermatozoa, the protamine complexes condense most of the DNA, but 5%–15% of the DNA remains associated to histones. This means that DNA methylation is not the only type of epigenetic information that the sperm cell may pass on to the oocyte. Also histone modifications may have an influence during embryonic development (57).

Finally, we studied the histone modifications H3K4me3, H3K9ac, H4K5ac, H4K8ac, H4K12ac and H4K16ac by immunohistochemistry. Most of the stage-specific histone modifications were not different from fertile adult controls. On the other hand, the stage-dependent expression of H4K5ac and H4K8ac, important for the histone-to-protamine exchange, was altered after SSCT. In elongated spermatids, however, the acetylation pattern was comparable to controls (58) (Fig. 6). Since the function of H4K5ac and H4K8ac in spermatogonia and spermatocytes is not known yet, it is interesting to carry out fundamental research into the exact functions of these modifications and their influence on fertility.

Another safety issue important for autologous transplantation in cancer patients is the risk of re-introducing malignant cells to the patient. Many pediatric malignancies are capable of metastasizing through the blood, introducing a potential risk of contamination of the collected testicular tissue. Jahnukainen et al. (59) demonstrated in a rat model that transplantation of as few as 20 leukemic cells could cause malignant recurrence in the recipient animal. In the human, the threshold number of malignant cells able to cause malignant relapse when transplanted to the testis is unknown. Therefore, it is of utterly importance to detect even the slightest contamination of the testicular tissue. In case of contamination, the isolation of SSCs from malignant cells before transplantation is necessary. Two research groups studied the use of MACS and/or FACS for depleting cancer cells from murine and human testicular cell suspensions. However, both reported insufficient depletion (60-62). Also cell selection by selective matrix adhesion was not sufficiently efficient (63).

**SSC CRYOPRESERVATION**

To safeguard the reproductive potential of young cancer patients, cryopreservation of testicular tissue containing SSCs is a potential fertility preservation strategy. This approach is preferred above cryopreservation of SSC suspensions because SSCs are kept in their original microenvironment. Evidence exists that the presence of the extracellular matrix and supporting cells is critical to germ cell survival and germ cell function (64). Any cryopreservation protocol should thus aim at preserving both the stem cells and their niche cells. Undoubtedly, cryopreservation of testicular tissue is a challenging task.
Figure 6. Histone modifications H4K5ac and H4K8ac in germ cells in control adult testis and testis obtained after SSCT in the mouse. The upper grid shows the cellular composition of the different stages according to the location of the cells in the tubules, with level 0 being the basal and level 3 the luminal side. For each spermatogenic stage, the percentage of tubules expressing the marker was determined. Statistical differences are shown by asterisks. In-spg: Intermediate spermatogonia; B-spg: Type B spermatogonia; PreL: preleptotene spermatocytes; L: leptotene spermatocytes; Z: zygotene spermatocytes; P: pachytene spermatocytes; D: diplotene spermatocytes; M2: second meiotic division; R spt: round spermatids; E spt: elongated spermatids.

The complexity of the tissue architecture demands optimal conditions for each cell type. Controlled slow freezing using dimethyl sulphoxide (DMSO) as cryoprotectant is a routinely used method to cryopreserve immature testicular tissue (65-68). In rodents, controlled slow freezing of prepubertal testicular tissue fragments has already led to the birth of healthy offspring (66). Two teams have published freezing protocols for human testicular tissue using controlled rate freezing. Kvist et al. reported a protocol which was initially developed for cryopreservation of human ovarian tissue, for the cryopreservation of testicular tissue in boys with cryptorchidism (70). Later, Keros et al. proposed a protocol for prepubertal testicular tissue, which was
originally developed for adult testicular tissue (71). Nevertheless, the drawback of controlled slow freezing is the need for expensive computerized equipment. Moreover, this freezing process consumes a lot of time and resources. Therefore, uncontrolled slow freezing was explored. As controlled freezing, uncontrolled freezing of prepubertal testicular tissue has been successfully used in different animal species, and has been fully validated in mice as a means to preserve the reproductive potential (72-75). Recently, in piglets and mice, vitrification was shown to yield similar results compared to slow freezing (76-78). Because both uncontrolled freezing and vitrification are inexpensive, convenient and fast executable protocols, these methods might be considered for human testicular tissue too (79).

**TESTICULAR TISSUE GRAFTING**

Testicular tissue grafting has been suggested as an alternative to SSCT. This technique involves the transplantation of SSCs within their original microenvironment and thus with their intact niches. Production of mature sperm was reported after grafting cryopreserved immature testicular tissue from mice and rabbits into the testis (66, 80). Progeny were born when rabbit sperm from the xenograft was used for ICSI (66). When compared with SSCT, intratesticular grafting resulted in a larger amount of donor-derived spermatogenesis (81). Cryopreservation of the grafts did not adversely affect the colonization efficiency and restoration of spermatogenesis. The next step should be the grafting of testicular tissue in higher species. It would be very appealing to know the applicability and efficiency of this technique in primates.

**CONCLUSIONS**

Now that cancer can be treated more successfully, the focus of the treatment is shifting more and more towards the quality of life after treatment. In adults and adolescents, semen banking or cryopreservation of testicular tissue before any treatment are valuable preventive measures in combination with techniques of assisted reproduction and especially ICSI. It is mainly the lack of awareness of both the oncologists and the male cancer patients that limits the preservation of the reproductive potential of the latter.

Two fertility preservation strategies are now being translated to the clinic: SSCT and testicular tissue grafting. Which fertility preservation and restoration strategy has to be chosen depends on the malignancy of the disease. Although grafting seems to be more efficient and is technically simpler than SSCT, it can only be done in patients with non-malignant diseases or non-metastasizing tumours. In case the risk for contaminating cells in the testis is substantial, the tissue should be digested and decontaminated before or after cryopreservation. SSCT would then be the only option for transplantation.

Although still purely experimental at this stage, both transplantation techniques may provide an adequate solution to preserve the progenitive capacity of pre-pubertal boys. The abovementioned findings are promising and important for its further clinical implementation. Cryobanking of testicular tissue from pre-pubertal boys is
already considered an acceptable strategy, analogous to cryobanking of ovarian cortex in young girls. Several institutions have already set up a testicular tissue bank. Patients with non-malignant hematological diseases and thus not at risk of malignant contamination of their testis tissue might be the first patients to be accepted for SSCT.

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LIST OF REFERENCES


